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ORIGINAL PAPER

Embryo culture is an efficient way to conserve a medicinally important endangered forest tree species *Strychnos potatorum*

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Abstract: The present study reports a protocol for germination of *Strychnos potatorum* (ver. Tel. Chilla) using zygotic embryo culture as an embryo rescue method. A 100% germination rate was obtained by culturing the embryos on full-strength Murashige and Skoog's medium (MS) containing 20 g/L sucrose in comparison to McCown and Lloyd's Woody Plant Medium (WPM). Germination rates decreased when the sucrose concentration was lower or higher than 20 $g \cdot L^{-1}$. WPM/MS medium containing glucose at levels 30, 20, 15 $g \cdot L^{-1}$ showed a smaller percentage of germination and at quarter strength, WPM/MS medium with glucose did not respond. Multiple shoot formation was found at 1.0–2.0 mg/L BAP; 3.0 mg/L Kn; 2.0 mg/L TDZ on MS medium with 20 $g \cdot L^{-1}$ sucrose. Germination rates improved when the embryos were placed upright (vertically) in the medium. The *in vitro* germinated seedlings were acclimatized in a walk-in-chamber and maintained in the green house with the survival rate of 65%–75%. These plants were transferred to the field and were found to be phenotypically normal, healthy and similar to donor plants. This protocol will be useful to overcome seed dormancy and for rapid multiplication and conservation of *S. potatorum* using zygotic embryo culture.

Keywords: endangered tree species; *Strychnos potatorum*; In vitro zygotic embryo culture; immobilization of zygotic embryos; embryo rescue

Introduction

Strychnos potatorum Linn. F (Fam: Loganiaceae) is commonly known as *Grape Strychnos* or Clearing nut tree or Katakam in Ayurvedic system of medicine. The ripe seeds are used for clearing muddy water. They are reported to be very effective as a coagulant. The seeds are also very effective when used with alum to remove the suspended impurities from coal washery wastes (Anonymous 1976). Polysaccharides present in the seeds are gummy in nature and hence are utilized in paper and textile industries (Adinolfi et al. 1994).

Various pharmaceutically and industrially important compounds were isolated from seeds of *S. potatorum,* including diaboline and its acetate (Singh et al. 1975), β-sitosterol, stigmasterol, oleanolic acid and its 3β acetate, saponions containing oleanolic acid (Singh & Dhar 1977), triterpines (Harkishan Singh & Kapoor 1975), manano galatans (Corsaro et al. 1995), norharmane, akuammidine, nor-C-fluroiocurane, bisnordihydrotoxiferine, 11-methoxy-henningsamine and 11- methoxy-12-hydroxydiaboline. According to Ayurveda, the seeds are acrid, alexipharmic and lithotriptic. They cure strangury, urinary discharges (Agharkar 1991). The seeds are used in hepatopathy, nephropathy, gastropathy, bronchitis, chronic diarrhea, dysentery, diabetes, and in eye diseases (Asima & Satyesh 2001). In Greek medicine, the seeds are used in liver and kidney complaints, gonorrhea, and for colic (Oudhia 2004). Traditionally the seed extract was used as contraceptive (Guptha et al. 2006).

S. potatorum is a fast disappearing species and is threatened due to indiscriminate collection and over exploitation as the plant is pharmaceutically, commercially and industrially important. The species is recognized as endangered and vulnerable plant species by Indian Forest Department and it requires "Certificate of Cultivation or Legal Procurement Certificate", mentioned in Ministry of Environment and Forests (MOEF) circular dt. 4.10.2000 (Sinha & Rawat 2008; Alliance for Natural Health International 2011). Commercial exploitation for production and conventional propagation is hampered due to poor viability (12 to 16 months) and a low rate of seed germination efficiency (30%–40%). Even clonal propagation is very poor (Kumar & Manoranjan 1992, Somashekhar & Manju Sharma 2002).

The fruit is eaten by birds, baboons and attacked by various fungi as well as bacteria. Being naturally propagated through

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seeds and hard wood cuttings, with long generation period, possibilities to improve the tree are limited (Somashekhar & Manju Sharma 2002).

In view of all these factors, the species has become endangered and there is no report on *in vitro* technique for multiplication and conservation of *S. potatorum.* The objective of our study was to determine the effect of different types of media, carbon source, plant growth regulators (PGRs), orientation of embryo and immobilization on enhancing germination percentage and plantlet establishment.

Materials and methods

Plant material

We collected fruits from open-pollinated trees during December-January 2009, from the Government Timber Depot (GTD) and Girijana Co-operative Corporation (GCC), Mahadevpur, Karimnagar, Andhra Pradesh, India. These fruits were washed under running tap water, treated with 15% (W/V) Bavistin solution for 5 min followed by 70% (v/v) ethanol (for 2 min). They were then washed several times with sterile distilled water. Later, the epicarp and mesocarp were removed to isolate the seeds. The seeds were stored in plastic bags at 4–15°C and used as root stock for *ex situ* conservation.

We washed the seeds under running tap water for 30 min, and disinfected the surface with commercial bleach (10% w/v Na-hypochlorite). The commercial bleach contains two drops of liquid detergent (Labolene, Qualigens, India) per 100 ml (20 min, with continuous agitation) followed by 70% ethanol for 30 s and washed with sterile distilled water five times. They were surface sterilized for second time with 0.1% $HgCl_2$ (w/v) for 2 min and then rinsed in sterile distilled water under aseptic conditions. These sterilized seeds were subjected to different treatments, including soaking in sterile distilled water (control), 10, 20, and 30 ppm filter sterilized Benzyl amino purine (BAP), Kinetin (Kn), Indole-3-acetic acid (IAA), 2, 4- dichlorophenoxy acetic acid (2, 4- D) and Gibberellic acid (GA_3) for three days. They were also treated with warm water for 10 min to increase the germination efficiency of embryos.

Culture media and culture conditions

For embryo rescue, mature zygotic embryos were isolated from ripened seeds under aseptic conditions after pretreatment and used as explants. To investigate the suitable culture media for rescue, zygotic embryos were inoculated on different culture media: MS medium (Murashige and Skoog 1962) and Woody Plant Medium (WPM, McCowan and Lloyd 1981) containing different concentrations of carbon source (sucrose/glucose). The pH of media was adjusted to 5.8 ± 0.1 either with 0.1N HCl or 0.1N NaOH before addition of 0.8% Difco-bacto agar and autoclaved at 121°C under15 lbs pressure for 15–20 min.

Zygotic embryos were cultured on (a) quarter, half and full strength MS and WPM media, each containing different concentrations of sucrose and glucose levels (15, 20, 30 $g \cdot L^{-1}$) and (b) MS medium containing 20 $g \cdot L^{-1}$ sucrose and different concentrations (0.5–5.0 $mg \cdot L^{-1}$) of cytokinins, BAP/Kn/TDZ, and auxins, IAA /2, 4- D.

Embryo orientation

The effect of embryo orientation on germination was studied by placing the embryos in vertical, horizontally and embedded positions; and also embryo without cotyledons (i.e. only with epical meristem) on MS medium containing 20 $g \cdot L^{-1}$ sucrose. Effect of immobilization on long-term storage (three years) of zygotic embryos was also studied by immobilizing them in sodium alginate (2%) mixed with 0.5 M $CaCl_2$ solution. These immobilized embryos were stored at 4°C and the germination efficiency was tested every six months (for a total of three years) by inoculating them on MS medium with 20 $g \cdot L^{-1}$ sucrose. All these cultures were maintained at 25 ± 2 °C, for a 16-hour photoperiod with the photon frequency of 40–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool white fluorescent lamps.

Plantlet establishment

The *in vitro* germinated plantlets were carefully removed from culture tubes and washed with sterile distilled water to remove the remains of medium. Later they were transferred to plastic pots containing vermiculite and maintained in a walk-in-chamber for four weeks. Later these plants were transferred to earthenware pots containing garden soil, and maintained in the greenhouse for 3 to 4 weeks and then transferred in to field.

Data analysis

The data on zygotic embryo germination was evaluated after four weeks of culture. Each experiment was repeated at least twice and each treatment consisted of three replicates with 50 explants. Significance was determined by calculating the mean and standard error.

Results

To determine the most efficient medium for embryo rescue in *S. potatorum,* the zygotic embryos were cultured on WPM and MS media containing different sucrose/glucose concentrations as the carbon source and also on MS medium fortified with different concentrations of PGRs.

Effect of nutrient media and carbon source

Significant differences was observed in the germination frequency of zygotic embryos among the nutrient media and carbon source used (Table 1). Zygotic embryos cultured on WPM and MS media with sucrose show better results than glucose.

Table 1. Effect of nutrient media and various concentrations of carbon source on zygotic embryo culture in *S. potatorum*.

Strength of medium	Carbon source (g·L^{-1})	% of germination	No. of days for germination[a]	Average shoot length[a, b](cm)	Average root length[a, b] (cm)
MS + Sucrose					
Full	30	92	9	3.5±0.32	8.8±0.31
Full	20	100	7	4.8±0.28	9.5±0.44
Full	15	88	8	1.5 ±0.13	7.4±0.20
Half	30	65	11	1.3±0.10	4.6±0.15
Half	20	74	9	1.2±0.07	6.4±0.35
Half	15	60	10	1.0±0.11	4.6±0.35
Quarter	30	10	11	1.2±0.07	2.0±0.33
Quarter	20	20	10	1.2±0.05	3.2±0.46
Quarter	15	15	12	1.0±0.11	3.7±0.33
WPM + Sucrose					
Full	30	43	8	2.91±0.36	5.28±0.22
Full	20	62	11	2.47±0.20	3.04±0.28
Full	15	35	11	1.40±0.17	4.22±0.29
Half	30	33	11	1.35±0.11	2.83±0.24
Half	20	45	12	1.16±0.06	2.64±0.25
Half	15	20	12	1.05±0.11	3.08±0.10
Quarter	30	20	13	1.35±0.10	1.71±0.19
Quarter	20	26	12	1.35±0.079	1.71±0.22
Quarter	15	15	14	1.40±0.13	2.13±0.12
MS+ Glucose					
Full	30	40	10	1.2±0.07	6.4±0.35
Full	20	32	12	1.3±0.10	4.6±0.15
Full	15	30	12	1.05±0.11	4.6±0.35
Half	30	25	12	1.05±0.11	3.1±0.10
Half	20	28	callus	--	--
Half	15	24	callus	--	--
Quarter	30	NR	--	--	--
Quarter	20	NR	--	--	--
Quarter	15	NR	--	--	--
WPM+ Glucose					
Full	30	42	10	1.2±0.07	6.4±0.35
Full	20	45	11	1.05±0.11	3.08±0.10
Full	15	30	12	1.05±0.11	4.6±0.35
Half	30	28	12	0.97±0.11	3.75±0.33
Half	20	32	callus	--	--
Half	15	NR	--	--	--
Quarter	30	NR	--	--	--
Quarter	20	NR	--	--	--
Quarter	15	NR	--	--	--

NR- No response. [a] Mean ± Standard Error. [b] After 4 weeks of germination.

In vitro zygotic embryo germination was started after seven days of culture. The first visible sign occurred after three days of inoculation as the embryos changed color, from whitish to yellowish. After 6 to 7 days of inoculation, radical elongation was started (Figs.1 a-b) and feathery cotyledons changed from yellowish to green after 7 to 8 days. Shoot emergence was observed after 9 to10 days of inoculation (Fig. 1c) and continued over the culture period to formation of the plantlet. Early zygotic embryo germination was observed on full-strength MS medium containing 20 g·L^{-1} sucrose.

Fig. 1 Zygotic embryo culture in *S. potatorum*. (a). zygotic embryo on full strength MS medium containing 20 g/L sucrose (vertically placed), (b). Radical elongation after 7 days of inoculation, (c & d). Shoot emergence and elongation after 2 and 4 weeks of inoculation respectively, (e& f). Plantlet development on MS containing 20 g/L sucrose supplemented with 2mg/L TDZ, 4mg/L TDZ respectively, (g & h). Acclimatization of plant in vermiculite containing pot, (i). Acclimatization of plant in earthenware pot containing garden soil, (j). Immobilized zygotic embryos.

Among the media and carbon source combinations, the maximum rate of germination (100%) was obtained on full-strength MS medium containing 20 g·L^{-1} sucrose with a 4.8 cm shoot length (Fig. 1d), followed by 30 g·L^{-1} (92% germination) and 15 g·L^{-1} (88% germination) sucrose with 3.5 cm and 1.5 cm shoot lengths, respectively. By comparison, WPM with 30 g·L^{-1} sucrose showed only 62% zygotic embryo germination (Table 1). Unlike the MS medium with sucrose, embryos germinated poorly on MS medium containing glucose and also on WPM medium containing sucrose and glucose. Apart from germination, embryos showed better response in terms of plant height, shoot length, root length, and leaf development, on MS medium with 20 g/L sucrose in comparison to other types of media used with different levels of carbon source.

Full-strength MS/WPM containing sucrose supported the proper development of seedlings whereas MS/WPM with glucose showed a poor response. The zygotic embryos cultured on quarter-strength MS and WPM containing low concentration of glucose did not respond. However, the embryos converted into callus on half-strength MS medium with 20 and 15 g·L^{-1} glucose and also on half-strength WPM with 20 g·L^{-1} glucose.

Effect of plant growth regulators

Zygotic embryos cultured on MS medium with 20 g·L^{-1} sucrose containing different concentrations of cytokinins (BAP/KN/TDZ) and auxins (IAA/2,4-D) individually had significant differences in growth and development (Table 2). The percentage of zygotic embryo germination reached maximum (100%) at 1.0 mg·L^{-1} BAP, 2 mg·L^{-1} and 3.0 mg·L^{-1} TDZ, among the PGRs tested. Cytokinins BAP, Kn and TDZ supported a high percentage of zygotic embryo germination in comparison to the auxins (IAA/2, 4-D) used. Among all the PGRs tested, BAP induced more

shoots (3.0 ± 0.23) showing superiority over other PGRs, followed by 2.0 mg·L^{-1} TDZ and 3.0 mg·L^{-1} Kn. The zygotic embryos cultured on MS medium fortified with 3.0–5.0 mg·L^{-1} IAA and 2, 4-D converted into callus after four weeks of inoculation. While at low concentrations of IAA and 2, 4-D up to 2 mg·L^{-1} supported for germination.

Table 2. Effect of different plant growth regulators on zygotic embryos of *S. potatorum* cultured on MS medium with 20 g·L^{-1} sucrose

Conc. Of PGR (mg·L^{-1})	No. of days for germination	% of response	No. of shoots/ seedling [a]	Average Shoot length (cms) [a]	Average root length (cms) [a]
BAP					
0.5	8	50	1.0±0.0	4.0±0.24	7.9±0.41
1	7	100	3.0±0.23	4.7±0.45	7.65±0.36
2	9	92	2.0±0.15	3.6±0.31	5.8±0.34
3	10	70	1.0±0.15	3.5±0.14	6.9±0.33
4	9	40	1.0±0.0	3.8±0.23	7.1±0.33
5	11	35	1.0±0.0	3.7±0.24	5.3±0.34
Kn					
0.5	11	40	1.0±0.0	3.7±0.34	6.3±0.44
1	10	65	1.0±0.0	5.2±0.27	5.6±0.32
2	9	71	1.0±0.0	4.4±0.20	5.7±0.34
3	7	90	2.0±015	5.5±0.37	7.0±0.42
4	8	55	1.0±0.15	4.3±0.16	6.1±0.26
5	10	30	1.0±0.0	4.8±0.23	6.4±0.37
TDZ					
0.5	11	45	1.0±0.0	5.25±0.22	6.6±0.37
1	9	52	1.0±0.0	6.47±0.20	7.1±0..41
2	7	100	2.0±0.15	7.37±0.22	9.1±0.33
3	8	100	1.0±0.08	8.79±0.14	8.2±0.31
4	7	50	1.0±0.31	9.05±0.46	9.7±0.32
5	10	28	1.2±0.11	8.64±0.11	8.2±0.31
IAA					
0.5	7	40	1.0±0.0	4.6±0.20	7.0±0.28
1	7	38	1.0±0.0	3.8±0.27	4.1±0.26
2	8	35	1.0±0.0	3.1±0.26	4.0±0.24
3	--	--	callus	--	--
4	--	--	Callus	--	--
5	--	--	callus	--	--
2, 4-D					
0.5	11	42	1.0±0.0	2.3±0.14	3.2±0.27
1	11	45	1.0±0.0	1.7±0.07	3.4±0.29
2	10	55	1.0±0.08	8.7±0.14	8.2±0.3
3	--	--	callus	--	--
4	--	--	callus	--	--
5	--	--	callus	--	--

[a] Mean ± Standard Error.

Data provided in Table 2 indicate that the type of PGR does affect zygotic embryo germination and multiple shoot induction. The results show that the cytokinins BAP (1.0–2.0 mg·L^{-1}) and TDZ (2.0 mg·L^{-1}) were found to be superior to other tested PGRs in terms of their ability to germinate with healthy seedlings in *S. potatorum*.

Embryo orientation

Zygotic embryo orientation also affected the germination percentage of embryos (Table 3). Zygotic embryos showed 100% germination in vertically placed position (Fig. 1a) whereas germination efficiency was reduced to 35% and 40% in the half-dipped and horizontally placed embryos respectively. The zygotic embryos without cotyledons showed a smaller percentage of germination compared to embryos with cotyledons and also converted into callus (Table 3). Only embryos placed vertically showed normal seedling development (5.4 ± 0.2 cm height) whereas half-dipped and horizontally placed zygotic embryos showed abnormal development in the form of delay in germination and absence of roots or shoots. The horizontally placed embryos showed stunted growth and rosette development of nodes.

Table 3. Effect of embryo orientation on MS+ 20 g/L sucrose on zygotic embryo culture in *S. potatorum*

Embryo orientation	% of germination	No. of days for germination	Seedling height(cm) [a, b]
vertical	100	6	5.4±0.2
Half-dipped	35	10	2.2±0.11
Horizontally placed	40	8	1.5±0.22 [c]
Without cotyledon	28	15	callus

[a] Mean ± Standard error, [b] after 3 weeks of inoculation, [c] Without roots.

Immobilization of embryos

Immobilized zygotic embryos (Fig. 1j) showed 49% of viability even up to 36 months, in comparison to embryos from seeds (control) (Table 4). The percentage of germination decreased as the storage period increased in the immobilized embryos. Zygotic embryos from seeds germinated up to 18 months. After that they didn't germinate, whereas the immobilized embryos could still germinate, even after three years.

Table 4. Effect of immobilization on viability of zygotic embryos in *S. potatorum*

Preservation period	% of germination in	
	Immobilized zygotic embryos	Embryos from seeds
6 months	100	98
12 months	96	60
18 months	85	20
24 months	65	0
30 months	58	0
36 months	49	0

Discussion

The results showed that MS medium containing sucrose played an important role in embryo germination and the MS medium supplemented with 20 g·L^{-1} sucrose was considered optimum for

embryo germination in *S. potatorum*. However, increases or decreases in sucrose concentration showed an effect on germination percentage and plantlet development in *S. potatorum* (Table 1). The absolute percentage of zygotic embryo germination was recorded on full-strength MS medium containing 20 $g \cdot L^{-1}$ sucrose. The maximum number of multiple shoot formation was also observed on MS medium containing 20 $g \cdot L^{-1}$ sucrose fortified with 1.0 $mg \cdot L^{-1}$ BAP.

Nutrients required by embryos vary depending on embryo age. Mature embryos can develop in a simple medium but embryos in the early developmental stages demand more complex medium (Monnier 1995; Collins & Grosser 1984; Johri & Rao 1984; Fisher- Iglesias & Neuhaus 2001). According to our observations, the mature zygotic embryos germinated on simple medium in *S. potatorum.* Similarly it was observed in an endangered forest tree *Givotia rottlorifomris* (Rambabu et al. 2006). Carbohydrates are very important for the culture of zygotic embryos because besides being a carbon source they regulate osmoticum of medium, a critical factor in zygotic embryo germination and development *in vitro*. Asif et al. (2001) also reported the importance of sucrose (5%) in MS medium for the culture of *Musa acuminata* ssp. *malaccensis* compared to other factors.

Full germination was obtained in *S. potatorum* in this study when embryos were cultured on MS medium fortified with 20 $g \cdot L^{-1}$ sucrose and supplemented with 1 mg/L BAP/2 $mg \cdot L^{-1}$ or 3 mg/L TDZ. Germination was also affected by embryo orientation. We found 100% germination when the meristematic end of embryos was placed upright in the medium as recorded in *Givotia rottleriformis* (Rambabu et al. 2006). The dormancy was also broken by enhancing the germination efficiency up to three years by using the immobilization technique in *S. potatorum*.

Conventional seedling development protocol is time consuming, laborious, and expensive. It will not fulfill the industrial or pharmaceutical need for *S. potatorum.* What is shown here is that *in vitro* embryo culture is a viable alternative to existing propagation techniques for breaking dormancy and ensuring a fairly uniform germination rate. This shift will make possible the rapid propagation and conservation of medicinally important forest tree species *S. potatorum.*

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